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Noninvolvement of Beauvericin in the Entomopathogenicity of *Beauveria bassiana*

F. R. CHAMPLIN AND E. A. GRULA*

Department of Cell, Molecular and Developmental Biology, Oklahoma State University, Stillwater, Oklahoma 74074

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Development of a microbiological autobiographic assay procedure permitted a detailed investigation of the possible role of beauvericin (a toxic ionophoric antibiotic produced by *Beauveria bassiana*) in the entomopathogenicity of *B. bassiana* against corn earworm (*Heliothis zea*) larvae. Analysis of spent media of *B. bassiana* and the hemolymph of infected and moribund larvae revealed that beauvericin was not present in a soluble form during the time that most (about 90%) larvae died of fungal infection (4 days). Intrahemocoelic injections of up to 6 μg of synthetic beauvericin failed to induce any deleterious effects. In addition, although methanol-soluble ionophores, such as valinomycin and bassianolide, were toxic to corn earworm larvae, no methanol-soluble toxin could be detected in the hemolymph of moribund larvae.

Beauveria bassiana, a member of the class Deuteromycetes (5), was first implicated as an insect pathogen in 1835 when Bassi showed it to be the causative agent of silkworm muscardine (13). The current interest in *B. bassiana* as a biological control agent of certain insect pests stems from the fact that it is the most common fungal isolate from dead and moribund insects in nature (11).

Although there can be very little doubt that *B. bassiana* is the most common fungal entomopathogen, precise information is lacking concerning the mechanism of pathogenicity. Evidence for the involvement of a soluble toxin(s) was provided by Dresner (4), who reported that the paralysis which resulted in three different insect genera upon exposure to germinating spores occurred in less time than it would have taken for the penetration of hyphae, as determined by histological techniques. Subsequent reports of extracellular substances toxic to insects have appeared (10, 17), but specific identification of the compounds responsible for toxicity and proof that they are indeed directly involved in pathogenicity in vivo are lacking.

The observation that silkworms infected by *B. bassiana* were never subsequently infected by bacteria (9) was the first indication that the fungus is capable of antibiotic production. A strain of *B. bassiana* has been shown to produce the antibiotic oosporein under certain growth conditions (16), and Hamill et al. isolated the ionophoric antibiotic beauvericin from mycelia and reported that it was toxic to brine shrimp and mosquito larvae (6). Several papers dealing with the chemical nature of beauvericin exist (3, 6, 7, 12, 14, 15), and the molecular structure is well established (Fig. 1).

Work in this laboratory has as its central theme the establishment of a molecular basis for the entomopathogenicity of *B. bassiana* using *Heliothis zea* (corn earworm) as the major target insect pest. The available literature contains little evidence either for or against the direct involvement of beauvericin in the infection of members of the order Lepidoptera. The purpose of this study was to develop a new assay procedure for beauvericin in order to assess its possible involvement in entomopathological processes. Currently, an assay using shrimp is the only biological test procedure available. The antibiotic nature of the molecule was exploited, permitting us to develop a specific, rapid, and readily reproducible autobiographic procedure utilizing a newly isolated *Bacillus* sp. from soil.

(A preliminary presentation of these results was made at the 78th Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 14-19 May 1978.)

MATERIALS AND METHODS

Fungal growth conditions. Cultures of *B. bassiana* were grown on Sabouraud dextrose agar (Difco Laboratories) slants at 25°C and maintained on the same medium at 2 to 4°C. One loopful of spores from 7- to 14-day cultures was used to inoculate a liquid medium consisting of 4% fructose and 1% Neopeptone (Difco; pH adjusted to 7.5 after autoclaving by using sterile 1.0 N NaOH). Each 250-ml Erlenmeyer flask contained 60 ml of medium, and cultures were incu-

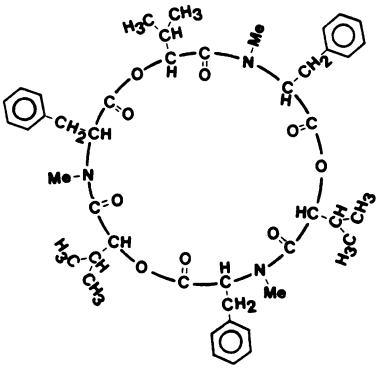

FIG. 1. *Molecular structure of beauvericin, a cyclic hexadepsipeptide ionophore consisting of three residues of D-α-hydroxyisovaleric acid and three residues of N-methyl-L-phenylalanine in alternating sequence.*

bated at 25°C on a rotary shaker for the times specified.

Larval growth conditions. Corn earworm larvae were reared on the corn, soy flour, and milk solids diet of Burton (1) at 25°C. Experiments were performed with early to middle fourth instar larvae (8 to 9 days old; weight, about 100 to 150 mg).

Autobiographic assay procedure. Whatman no. 1 paper chromatograms of samples to be checked for the presence of beauvericin were developed in a solvent system consisting of methanol, water, and formic acid (30:45:25) in which synthetic beauvericin migrated to an R_f of about 0.90. Running time for the solvent was about 1.5 h at 25°C. Assay plates were prepared by adding 200 ml of liquid Sabouraud dextrose agar seeded with 6 ml of the *Bacillus* sp. suspension (about 2.5×10^6 viable cells per ml) to a sterile Pyrex plate (22 by 34 cm; baking plate) covered with aluminum foil. After solidification of the agar, paper chromatograms, air-dried for about 3 h in an exhaust hood, were laid over the solid medium and permitted to remain during incubation of the plates at 25°C. Zones of growth inhibition underneath or around specific areas of the paper chromatograms could be observed after incubation for 18 to 24 h. A typical bacterial response is shown in Fig. 2. Although not clearly defined, a hazy zone of clearing can be seen with as little as 0.5 μg of beauvericin. Morphological and biochemical characteristics of the *Bacillus* sp. used are given in Table 1. This bacterium was selected from over 600 soil isolates on the basis of its being judged most sensitive to synthetic beauvericin. None of the descriptions given in either the seventh or eighth editions of *Bergey's Manual of Determinative Bacteriology* fits this organism.

Analysis of spent growth medium. Broth cultures of *B. bassiana* were removed from the shaker at selected times, and fungal growth was removed by centrifugation at 8,000 rpm (Sorvall RC-2) for 20 min followed by membrane filtration (0.45 μm; Millipore Corp.). The clear and sterile spent medium was then distributed in 10-ml portions and lyophilized. An initial fractionation was carried out as follows. Methanol (1 ml) was added to a lyophilized sample; this was agitated on a Vortex mixer for 1 min at 25°C, after which it was centrifuged (International Clinical Centrifuge; model CL) and the methanol-soluble material was poured off. Water (1 ml) was added to the residue (water-soluble fraction). Both fractions were then deposited (5 to 40 μl) on Whatman no. 1 paper for chromatography and subsequent autobiographic testing.

Analysis of hemolymph of infected larvae. Early to middle fourth instar corn earworm larvae

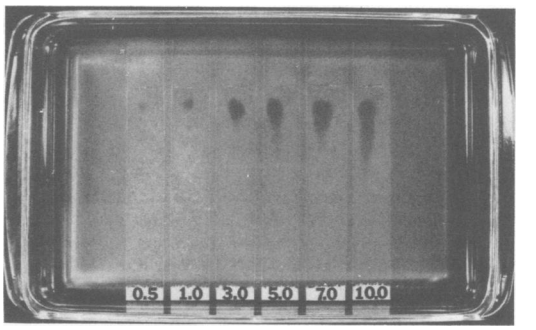

FIG. 2. *Autobiographic assay plate showing zones of bacterial growth inhibition by various concentrations of synthetic beauvericin at R_f 0.90. Values are given in micrograms.*

TABLE 1. *Morphological and biochemical characteristics of the Bacillus sp. used in the autobiographic assay*

Parameter[a]	Results
Gram stain	Positive, stains unevenly
Motility	+
Spore formation[b]	Terminal, oval, cell distended
Production of acid with glucose	−
Nitrate reduction to nitrite	−
Starch hydrolysis	+
Tributyrin hydrolysis	+
Gelatin hydrolysis	+
Catalase production	+
Urease production	−
Growth with citrate	+
Growth with allantoin	−
Indole	−
Methyl red	−
Voges-Proskauer	−
Litmus milk[c]	−
Chromogenesis	Pale yellow (water insoluble)
Growth in Sabouraud dextrose broth	+

[a] Biochemical tests were read after incubation for 24 h at 25°C with the exception of those noted.

[b] Spore formation occurred by 36 h on tryptic soy agar (Difco) at 25°C.

[c] There was no detectable change in pH or curd formation in litmus milk within 72 h.

were infected by direct intrahemocoelic injection of 1 μl of phosphate-buffered saline containing *B. bassiana* spores using the injection procedure of Cheung et al. (2). The infective doses used ranged from 360 to 2,750 viable spores per larva. Larvae were then incubated at 25°C until the symptoms of infection became advanced (usually 4 days), at which time the hemolymph was collected and pooled (2). Control hemolymph was obtained by injecting sterile phosphate-buffered saline into larvae and incubating under the same conditions as test larvae. Pooled hemolymph samples were then checked for the presence of beauvericin after paper chromatography of whole hemolymph (cells removed by centrifugation) and methanol extracts of lyophilized whole hemolymph.

Injection of ionophoric antibiotics. Intrahemocoelic injections of beauvericin, bassianolide, and valinomycin (Sigma Chemical Co.) were performed by using ethanol since this solvent is not toxic to fourth instar larvae when injected in 1-μl amounts. Methanol, a solvent often used for the solubilization of ionophoric antibiotics, was found to be toxic to fourth instar larvae (about 50% kills were obtained with the injection of 1 μl of methanol per larva).

RESULTS AND DISCUSSION

Analysis for beauvericin in spent growth media of *B. bassiana*. With our conditions of infection, death of corn earworm larvae is generally about 90% complete after 4 days (P. Y. K. Cheung and E. A. Grula, unpublished data). Autobiographic analysis of spent growth media for beauvericin after incubation of *B. bassiana* for 3, 4, and 5 days failed to demonstrate the presence of any beauvericin. Growth medium samples were concentrated by a factor of 10 in the course of sample preparation, and further concentration occurred when depositions were made for paper chromatography. Therefore, if beauvericin is released into the medium during growth of *B. bassiana*, it must be present in extremely low concentrations, considering the sensitivity of our assay (micrograms). Such results make it very unlikely that secretion of beauvericin is directly involved in the pathogenicity of *B. bassiana*.

Analysis for beauvericin in the hemolymph of moribund larvae. Although beauvericin could not be detected as a soluble entity in spent growth media, the possibility exists that it is produced in the hemolymph, where more favorable conditions might be present for the growth of the fungus. To test this possibility, hemolymph from fourth instar larvae was analyzed 4 days after the injection of viable spores. The mean body weight of larvae at this stage of infection (just before death) was only about one-half that of healthy control larvae. At no time could any beauvericin be detected in the hemolymph of such infected and moribund larvae.

Although no beauvericin could be detected in infected hemolymph, a zone of bacterial growth inhibition was apparent at the origin of chromatograms; however, such inhibition was evident in the hemolymph of both healthy and infected larvae. Apparently, the responsible material is a water-soluble component of relatively high molecular weight, because it remains at the origin in both polar and relatively nonpolar paper chromatographic solvent systems.

Search for methanol-soluble toxic components in the hemolymph of moribund larvae. Because of the nonpolar surface characteristics of ionophoric molecules, such compounds are preferentially soluble in solvents such as methanol. To detemine whether any methanol-soluble compounds toxic to corn earworm larvae are produced during an active infection with *B. bassiana*, the following experiment was done. Hemolymph from infected larvae (25/experiment; three experiments) was obtained just before death, and 1-ml portions were lyophilized. Lyophilized material was then extracted with 0.2 ml of methanol (25°C for 10 min), and the insoluble material remaining was removed by centrifugation. The methanol extract was then dried at 25°C under N_2, redissolved in 0.2 ml of ethanol (to avoid methanol toxicity), and injected (1 μl/larva) into 20 healthy corn earworm larvae in each of three experiments. Even though our procedure resulted in a better than fivefold increase in the concentration of hemolymph, neither control nor infected hemolymph extracts had any deleterious effect upon healthy larvae, nor did paper chromatograms of the infected hemolymph extract contain detectable beauvericin. This leads us to conclude that the pathogenicity of *B. bassiana* does not directly involve the release of any nonpolar ionophore-like toxin.

Direct injection of ionophores. In addition to beauvericin, several ionophores (bassianolide, valinomycin, nigericin, and A23187) were injected directly into the hemolymph of early fourth instar larvae to determine whether known levels of such compounds are indeed toxic to corn earworms. Representative data obtained with those ionophores, which are structurally related to beauvericin, are given in Table 2. Each ionophore was injected into 100 larvae, and the results are given as the percentage of larvae affected at various times postinjection. Synthetic beauvericin (up to 6 μg, an amount readily detected in our assay system) was not toxic even upon direct injection. The volume of early fourth instar larvae varies from about 100 to 150 μl; thus, an injection of 6 μg of beauvericin represents a 4 to 6% solution in vivo.

Bassianolide, a cyclic depsipeptide recently

TABLE 2. *Intrahemocoelic injections of beauvericin and the structurally related ionophores valinomycin and bassianolide into fourth instar H. zea*[a]

Compound injected	Amt injected (μg)	Concn (M)	% of larvae affected at the following times (h) postinjection:[b]			
			6	24	48	72
Ethanol (control)[c]			5.4 (2–11)	0 (0–4)	0 (0–4)	0 (0–4)
Beauvericin	1.0	1.28×10^{-3}	6.7 (3–14)	0 (0–4)	0 (0–4)	0 (0–4)
Beauvericin	6.0	3.83×10^{-3}	10 (6–16)	0 (0–4)	0 (0–4)	0 (0–4)
Bassianolide	1.0	1.10×10^{-3}	75 (66–84)	10 (6–16)	5 (2–10)	0 (0–4)
Bassianolide	6.0	6.61×10^{-3}	85 (78–92)	70 (60–79)	5 (2–10)	0 (0–4)
Valinomycin	1.0	9.00×10^{-4}	100 (96–100)	100 (96–100)	100 (96–100)	100 (96–100)

[a] All compounds were injected in 1 μl of ethanol, except the 6-μg amount of beauvericin, which was injected in 2 μl.

[b] Percentage of larvae paralyzed, atonic, or dead at various times postinjection. All larvae were killed within 2 to 6 h with valinomycin, whereas bassianolide only induced atonic symptoms from which, as indicated, all larvae eventually recovered. The values in parentheses represent the binomial confidence limits obtained from a sampling size of 100 larvae at a 95% confidence level.

[c] The control was a 1.0-μl injection of ethanol.

isolated from the mycelia of *B. bassiana* by Kanaoka et al. (8), has been shown to induce atony in larvae of *Bombyx mori* when 2-μg/larva injections are made; doses greater than 5 μg/larva were reported to be lethal (8). These investigators also reported that beauvericin, at concentrations of up to 100 μg/larva, had no effect on *B. mori*. We have observed that 1 and 6 μg of bassianolide totally immobilize all corn earworm larvae within 2 h. The majority remain atonic for up to approximately 24 h with 1-μg injections and for 48 to 72 h with 6-μg injections, after which recovery appears to be complete. In comparison to beauvericin and bassianolide, as little as 1 μg of valinomycin killed 100% of all larvae injected within 2 to 6 h after injection. Although injected in methanol (1 μg in 1 μl), nigericin and A23187 produced no greater kills than those obtained with the methanol solvent alone. It is therefore apparent that some ionophores can exert deleterious effects upon corn earworm larvae; however, beauvericin (at significantly higher molar concentrations than either valinomycin or bassianolide) has no such effects. Interestingly, bassianolide is toxic to corn earworm larvae and beauvericin is not; however, beauvericin is toxic to our *Bacillus* sp., whereas bassianolide exhibits no observable toxicity against this bacterium.

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